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Genetic Mapping of Loci Associated with Seed Phytic Acid Content in CX1834-1-2 Soybean

D. R. Walker,* A. M. Scaboo, V. R. Pantalone, J. R. Wilcox, and H. R. Boerma

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] seed phosphorus is stored primarily as phytic acid, a form in which it is unavailable to monogastric mammals and birds. Because of the nutritional and environmental problems caused by phytic acid, development of cultivars with low phytic acid (*lpa*) mutations has become an important objective in many soybean breeding programs. Information about the inheritance of the low phytate trait would facilitate these efforts. The objectives of the current research were (i) to map low phytate loci in populations derived from the *lpa* mutant line CX1834-1-2, (ii) to identify closely linked molecular markers, and (iii) to characterize inheritance of the trait. We identified two loci associated with the low phytate phenotype of CX1834-1-2 and discovered an epistatic interaction between the loci. A locus on linkage group (LG) N near Satt237 accounted for 41% of the observed variation in seed inorganic phosphorous (P_i) levels, which are inversely correlated with phytate levels in plants carrying the *lpa* mutation. Another locus near Satt527 on LG L explained 11% of the variation in seed P_i levels, and an interaction between the LG L and N loci accounted for an additional 8 to 11%. The loci on LG N and LG L are probably the previously designated *pha1* and *pha2* loci.

DEVELOPMENT of low phytate cultivars has become an important objective in soybean breeding programs. Phytate, a mixed cation salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate), is the form in which 67 to 77% of the phosphorus (P) in soybean seed is stored (Raboy et al., 1984). Phytate P is nutritionally unavailable to monogastric animals such as poultry, swine, and humans (Erdman, 1979; Larson, 1998). In addition, phytic acid chelates cations such as potassium, magnesium, iron, zinc, and calcium, thereby reducing their nutritional availability (Erdman, 1981; McCance and Widdowson, 1935). Diet rations fed to poultry and swine are typically supplemented either with phytase, which catalyzes the stepwise removal of phosphate from phytate (Lei and Porres, 2003), or with inorganic phosphate (P_i) to increase P availability (Adeola et al., 1995). Eutrification of surface water can result from runoff and leaching of excessive P in soils to which livestock or poultry manure has been applied (Ertl et al., 1998). Excretion of phytate P by nonruminant animals is therefore potentially detrimental to the environment, and supplementation of soy meal with P_i can exacerbate this problem.

Low phytic acid (*lpa*) mutations induced by treatment of seeds with ethyl methanesulfonate (EMS) have been used to lower phytate levels in soybean (Wilcox et al., 2000; Hitz et al., 2002), barley (*Hordeum vulgare* L.; Larson et al., 1998; Rasmussen and Hatzack, 1998), maize (*Zea mays* L.; Raboy and Gerbasi, 1996; Raboy et al., 2000), rice (*Oryza sativa* L.; Larson et al., 2000), and wheat (*Triticum aestivum* L.; Guttieri et al., 2004). Wilcox et al. (2000) developed *lpa* mutants of the soybean breeding line CX1515-4 by treating seeds with EMS and then testing M3 seeds for elevated levels of P_i. Two M2 plants, M153 and M766, were identified as having *lpa* mutations resulting in phenotypes in which the increase in P_i was associated with an equivalent decrease in phytic acid, as had been observed in the *lpa1-1* mutants of maize and barley (Larson et al., 1998). In the maize *lpa1-1* mutant, a 60% reduction in phytic acid was accompanied by a molar-equivalent increase in P_i (Ertl et al., 1998; Raboy and Gerbasi, 1996). These single-gene mutations are recessive, and translocation of gene products or metabolites in heterozygous (*Lpa*/*lpa*) barley plants does not complement the loss of gene function in their homozygous mutant (*lpa*/*lpa*) seeds (Larson et al., 1998). In the seeds of M6 progenies descended from the mutant soybean line M153-1-4, P_i accounted for 60 to 70% of the sum total of phytate P and inorganic P, as compared with only 15% in the cultivar Athow (Wilcox et al., 2000). Overall, approximately 75% of seed total P in M153-1-4 should be available to monogastric animals, whereas only about 25% of the total P in seeds from soybean with normal phytate levels would be available.

At the Univ. of Georgia, we began in 2001 to investigate the inheritance of the low phytate trait in soybean with the assumption, based on data from Wilcox et al. (2000), that a single locus with a mutated allele was responsible for the low phytate phenotype. The *lpa* mutations in maize, barley, and rice had each been mapped to a single locus (Larson et al., 1998, 2000; Raboy et al., 2000). These results and the low mutation rates expected from EMS mutagenesis suggested that the low phytate phenotypes in the mutant soybean lines of Wilcox et al. (2000) were also the result of a mutation in a single gene. We therefore expected to be able to map this locus using small $F_{2:3}$ populations from Athow × M153-1-4-6-15-3 (37 F_2 individuals) and Savoy × M153-1-4-6-29-2 (40 F_2 individuals), and a BC_1F_2 population of 94 individuals

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Abbreviations: BSA, bulked segregant analysis; CIM, composite interval mapping; IM, interval mapping; LG, linkage group; MG, maturity group; P_i, inorganic phosphate; RIL, recombinant inbred line; SSR, simple sequence repeat.

from [(Savoy × M153-1-4-6-29) × Savoy]. Assuming segregation at a single locus, seed from approximately 25% of the F_2 plants, and about 12.5% of the F_2 progeny of BC_1F_1 individuals would be expected to have seed P_i and phytate levels equivalent to the low-phytate parent. When a much lower than expected percentage of the individuals in these populations were found to have the *lpa* phenotype, and a large number of individuals were observed with phenotypes intermediate between those of the parents, we began to suspect that inheritance of the *lpa* trait in soybean was more complex than originally assumed. At that point, we switched our mapping efforts to the larger populations described in this paper. An independent investigation of the inheritance of the low phytate trait from CX1834-1-2 was begun at the University of Tennessee in 2002. The data presented in this paper are the result of a collaboration which subsequently developed between researchers at the Universities of Tennessee and Georgia.

Efforts to develop soybean cultivars with reduced phytic acid levels will be facilitated by knowledge about the locations and contributions of phytic acid loci and by identification of DNA markers that can be used for marker-assisted selection (MAS). The objectives of the current research were (i) to map low phytate loci in CX1834-1-2, (ii) to identify closely linked molecular markers, and (iii) to characterize the inheritance of the trait.

MATERIALS AND METHODS

Plant Material

Most of the mapping work reported here was done with a composite F_2 population of 'AGS Boggs-RR' × CX1834-1-2 developed at the Univ. of Georgia. This mapping population was composed of 226 F_2 individuals from six different F_1 plants. AGS Boggs-RR (hereafter referred to as "Boggs-RR") is a glyphosate-tolerant, normal phytate isoline of the maturity group (MG) VI cultivar Boggs (Boerma et al., 2000). CX1834-1-2 is a $F_{3:5}$ low phytate line which was developed by the USDA and Purdue Univ. from a cross of 'Athow' × M153-1-4-6-14. Athow is an MG III cultivar (Wilcox and Abney, 1997), and M153-1-4-6-14 is descended from the original M153 low phytate mutant of Wilcox et al. (2000). F_2 seeds were planted in a greenhouse 31 July 2001 in 1-L Styrofoam cups containing Fafard 2 Mix (Conrad Fafard, Inc., Agawam, MA). After the F_2 plants had become established, each was fertilized weekly with approximately 6 mg N, 3 mg P, and 5 mg K.

The locations and effects of loci putatively associated with low phytate levels in the Boggs-RR × CX1834-1-2 population were later confirmed in two other independent populations. One was a subset of an $F_{2:3}$ population from 'Hartz H7242 RR' × CX1834-1-2 developed at the Univ. of Georgia. Hartz H7242 RR (hereafter referred to as "Benning-RR") is a glyphosate-tolerant near-isoline of the MG VII cultivar Benning (Boerma et al., 1997). The 153 individuals in this subset were selected on the basis of homozygosity for CX1834-1-2 alleles at the linkage group (LG) N markers Satt237, Satt339, and Satt255. Thus the genotype at the LG N phytate locus did not contribute to the genetic source of variation in P_i levels in this population. The second confirmation population consisted of a set of 187 F_5–derived random recombinant inbred lines (RILs) from the cross '5601T' × CX1834-1-2, which were developed at the Univ. of Tennessee using single-seed descent. 5601T is a Univ. of Tennessee F_6–derived MG V cultivar derived from the cross of 'Hutcheson' × TN89-39 (Pantalone et al., 2003). The RILs and their parents were grown in 3-m-long single row plots with three replications of a randomized complete block. The plots were planted in 2003 on a Sequatchie Fine Sandy Loam with a 0 to 2% slope at the Knoxville Experiment Station in Knoxville, TN. Soil tests indicated that 90.2 kg ha^{-1} P was available in the field in which the plots were grown.

Molecular Analyses

University of Georgia

DNA was extracted from pulverized leaf or seed tissue of the Boggs-RR × CX1834-1-2 and Benning-RR × CX1834-1-2 populations by a CTAB extraction method (Keim et al., 1988). F_2 genotypes were reconstructed by pooling leaf or seed tissue from eight $F_{2:3}$ siblings before DNA extraction. PCR and polyacrylamide electrophoresis protocols were the same as those described by Li et al. (2001), except that GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) and PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA) thermal cyclers were used. PCR amplicons were separated by polyacrylamide gel electrophoresis on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Simple sequence repeat (SSR) markers were used in three different approaches to search for phytate loci in the Boggs-RR × CX1834-1-2 population: (i) bulked segregant analysis (BSA; Michelmore et al., 1991), (ii) a modified BSA method which is described later, and (iii) a whole-genome scan of a subset of 94 random individuals. For the BSA and the modified BSA, 180 markers were tested for polymorphisms between the two parents. Additional markers were added for the whole-genome scan, and 318 markers altogether were tested for polymorphisms. These markers were chosen on the basis of the availability of fluorescently labeled primer sets and on their estimated locations on the integrated linkage maps of Cregan et al. (1999) and later Song et al. (2004). For the whole-genome scan, we tried to find at least one reliable polymorphic marker every 15 to 20 cM along each linkage group. Genotype data were then used to search for regions of the genome putatively associated with variation in P_i levels. Data from markers which had unusual segregation ratios or whose genotypes did not correspond with those of flanking markers on a linkage group were not used.

University of Tennessee

For the 5601T × CX1834-1-2 population, DNA was extracted from pulverized tissue of four to five leaves, each collected from a random $F_{5:7}$ plant, with Qiagen DNeasy Plant Kits (Valencia, CA). DNA was also extracted from parent plants included as controls. PCR reactions were conducted with a ThermoHybaid multi-block system (Franklin, MA), as described by Hyten et al. (2004b). Amplicons for LG L and N markers that had been identified through the previous Univ. of Georgia investigations were separated by capillary electrophoresis on a Beckman-Coulter CEQ 8000 Genetic Analysis System (Fullerton, CA).

P_i Assays

Since the phenotype of the M153 mutant ancestor of CX1834-1-2 had previously been characterized as being similar to the *lpa1-1*-type mutants in maize, an inverse relationship between phytic acid and P_i levels was assumed (Raboy et al., 2000; Wilcox et al., 2000). This inverse relationship, with no

difference in total P, has also been observed in three independent populations derived from crosses between normal phytate parents and CX1834-1-6, a sister line of CX1834-1-2 (Oltmans et al., 2005). We therefore expected most of the nonphytate P in CX1834-1-2 and its progenies to be in the form of P_i. Seed P_i levels in the Boggs-RR × CX1834-1-2 population were determined by a modified version of a colorimetric assay developed by Raboy et al. (2000), which was adapted from the assay described by Chen et al. (1956). In this modified assay, eight randomly chosen seeds from each F_2 plant were placed in small glassine envelopes and pulverized in bulk into particles ≤1.5 mm in size with a hammer. Three samples of the crushed seed tissue in each envelope were then transferred to 2.0-mL microcentrifuge tubes for P_i extraction. Samples of 150 mg of tissue were thoroughly mixed with extraction buffer [12.5% trichloroacetic acid (TCA) and 25 m*M* $MgCl_2$] at a ratio of 10 μL buffer per mg of tissue, and incubated overnight (~15 h) at 4°C. If there was insufficient tissue for 150-mg samples, 100 mg of tissue was used, but the tissue to buffer ratio remained the same. The samples were then vortexed and allowed to settle for 30 min before aliquots of extract solution were collected.

For the colorimetric assays of the Boggs-RR × CX1834-1-2 and Benning-RR × CX1834-1-2 populations, 5 μL of sample extract was diluted with 95 μL of filtered H_2O. The dilution was increased from that of Raboy's original protocol (10 μL extract diluted with 90 μL water) to keep the reaction intensities of CX1834-1-2 and other high-P_i samples within the range of the five P standards (0.0, 0.15, 0.46, 0.93, and 1.39 μg P) used as controls. The diluted samples (100-μL total volume) were then mixed with 100 μL of Chen's Reagent, which consists of 1 volume 6 N H_2SO_4: 1 volume 0.02 *M* ammonium molybdate: 1 volume 10% ascorbic acid: 2 volumes water (Chen et al., 1956). The reaction was allowed to proceed at ambient temperature for 1 h, after which sample reaction intensities were either scored visually or measured with a PowerWave microplate spectrophotometer with KC4 data collection and analysis software (Bio-Tek Instruments, Inc., Winooski, VT). For the spectrophotometer readings, the wavelength was set at 645 nm, which was as close as we were able to get to the 882 nm wavelength used by Wilcox et al. (2000) with equipment available at the Univ. of Georgia. Reactions of a subset of the population consisting of 166 F_2 individuals were initially measured with the spectrophotometer. Sample reactions were also rated visually by comparing the blue color intensities to those of the five P standards. Reactions were scored from 1.0 (no reaction) to 5.0 (dark blue and similar to the 1.39 μg P standard), with increments of 0.5. Since absorbance readings correlated well with visual ratings of reaction intensity, visual ratings were subsequently used at the Univ. of Georgia to avoid damaging the spectrophotometer with the sulfuric acid in the Chen's Reagent. The visual ratings were considered to be a semiquantitative measurement of the P_i levels in the $F_{2:3}$ seeds. An estimate of the relative P_i content of each F_2 was obtained by assaying two aliquots from each of three independent extractions from $F_{2:3}$ seed and calculating the mean of the six visual ratings (CV = 12%).

The procedures used at the Univ. of Tennessee to assay P_i levels in the 5601T × CX1834-1-2 population were similar to those used at the Univ. of Georgia, with a few minor differences. Approximately 30 g of seed from each $F_{5:7}$ line were ground for 20 s in a Knifetec 1095 Sample Mill (FOSS Tecator, Hogana, Sweden). Next, 100-mg samples of this tissue were mixed with 1 mL of extraction buffer, vortexed, and incubated overnight at 4°C. Afterward, the samples were vortexed and allowed to settle for 5 min before 200-μL aliquots of the extraction solution were transferred to a 96-well plate and centrifuged at about 2520 *g* for 3 min. Three 10-μL subsamples of each extract were transferred to another 96-well plate and then assayed with Chen's Reagent to obtain a mean estimate of P_i levels for each individual in the population. P_i concentrations were determined with a Bio-Tek PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) set at a wavelength of 882 nm. Eight standards were used to obtain a standard curve, from which sample concentrations were estimated. In addition to the five standards used at the Univ. of Georgia, standards representing 1.86, 2.32, and 2.64 μg P were included. This made it possible to use the same sample dilution ratio (10 μL P_i extract solution + 90 μL H_2O) used by Raboy (2000). The P_i concentrations for these RILs represent means averaged across subsamples and the three field replications.

Mapping

A BSA approach (Michelmore et al., 1991) was taken initially to search for markers associated with P_i variation in the Boggs-RR × CX1834-1-2 population. Bulks of DNA from high P_i (HIP)/low phytate plants and low P_i (LIP)/normal phytate plants were constructed on the basis of mean visual ratings from the assays. Two HIP bulks, HIP 1 and HIP 2, were each composed of DNA from seven F_2 plants whose seed extracts had produced a dark blue color (visual ratings ranged from 3.8–4.0), similar in intensity to the mean of the CX1834-1-2 samples (3.83). Because of the small number of F_2 plants with dark blue reactions, five high-P_i F_2 plants were included in both of the HIP bulks (i.e., DNA from these five plants was present in both HIP bulks). The LIP 1 and LIP 2 bulks were composed of DNA from seven individuals whose seed extracts produced little or no reaction (visual ratings from 1.2–1.5), similar to those of Boggs-RR (1.33). Since many samples produced no reaction, each LIP bulk was composed of a unique set of individuals. One hundred eighty polymorphic SSR markers dispersed relatively evenly among the 20 linkage groups were used to amplify DNA from Boggs-RR, CX1834-1-2, and the four bulks to allow us to search for amplicon patterns suggestive of a marker-phenotype association.

In the modified BSA method used to search for additional loci following detection of the LG N phytate locus, individuals from the Boggs-RR × CX1834-1-2 population were identified that were homozygous for the CX1834-1-2 allele at three markers tightly linked to the phytate locus on LG N (Satt237, Satt339, and GMABAB). Assay reaction phenotypes of $F_{2:3}$ seed from this subset of the population ranged from dark blue to a light blue that was intermediate between the reactions of the parents. A medium P_i (MIP) bulk and a high P_i (HIP) bulk were each created by pooling DNA from eight F_2 individuals with either intermediate (visual ratings 1.4–2.4) or high P_i (visual ratings 3.0–4.2), respectively. These bulks were then screened with 63 known polymorphic markers in a search for amplicon patterns suggesting marker linkage to a phytate locus.

A genome scan approach was also used to search for additional phytate loci. This was initiated by genotyping a subset of 94 F_2 individuals from the Boggs-RR × CX1834-1-2 population at polymorphic marker loci distributed across the 20 linkage groups. All 226 F_2 plants from this population were eventually genotyped at seven marker loci on LG N (spanning ~47 cM) and five on LG L (spanning ~51 cM) to estimate the location of the phytate loci on those two linkage groups. Before genotyping the Benning-RR × CX1834-1-2 $F_{2:3}$ population, we tested 32 SSR markers from LGs L and N for polymorphisms between the two parents. Ten polymorphic markers were then used to genotype the 153 F_2 individuals that were homozygous for the CX1834-1-2 alleles at Satt237 and Satt339 on LG N. Five markers on LG L spanned a ~92-cM

region, and five on LG N spanned approximately 52 cM (Song et al., 2004).

Marker-phenotype associations in the Boggs-RR × CX1834-1-2 population were preliminarily tested by single-factor analysis of variance with PROC GLM (SAS Institute, Cary, NC). The estimated position of a phytate locus on LG N was initially mapped using 184 F_2 individuals from this population. Seven SSR markers on LG N were used in marker regression analysis, interval mapping (IM), and composite interval mapping (CIM) with the Map Manager QTX program developed at the Roswell Park Cancer Institute (Chmielewicz and Manly, 2002). The likelihood ratio statistics calculated by this program were divided by 4.6 to obtain LOD score equivalents. CIM was conducted following IM to test for the presence of additional QTL elsewhere on the LG. The most significant marker from the regression analysis was used as a background marker in the CIM analysis. For both IM and CIM, genome-wide significance thresholds ($\alpha = 0.001$) were calculated empirically by conducting chance association tests on 1000 permutations of the genotypic and phenotypic data sets (Churchill and Doerge, 1994). These same analysis programs and methods were also used to map the LG L locus in the Boggs-RR × CX1834-1-2 population. QTL Cartographer (Basten et al., 1995) was used for IM and CIM of the LG N locus early in the mapping study to confirm the results from Map Manager QTX. Marker regression analysis, IM, and CIM were also run on the Benning-RR × CX1834-1-2 population using Map Manager QTX. Both PROC GLM and Map Manager QTX were used to test for epistasis. PROC GLM was used to analyze data from the 5601T × CX1834-1-2 RIL population.

RESULTS AND DISCUSSION

Data from our studies indicated that inheritance of the low phytate phenotype from soybean CX1834-1-2 is quantitative (Fig. 1) and that loci on LGs N and L are associated with variation in seed phytic acid content, which is inversely related to seed P_i content in *lpa* mutants (Table 1). If inheritance of the low phytate trait involved a single gene, approximately 25% of the individuals in the Boggs-RR × CX1834-1-2 mapping population should have produced a color reaction similar to that of CX1834-1-2. However, in a preliminary P_i assay of 166 F_2 plants from this population, a lower than expected number of individuals produced a reaction similar to that of CX1834-1-2 checks, which averaged 45 to 50 relative concentration units, on the basis of absorbance (Fig. 1). Mean P_i concentrations among the progeny ranged from 0 to 60 units, but only 6.6% of the progeny had readings in the 30 to 60 range (Fig. 1). Mean visual assay ratings for all 226 individuals in this population ranged from 1.00 to 4.25 on a rating scale of 1.00 (no reaction) to 5.00 (dark blue, and equivalent to the 1.39 μg P standard), but most were more similar to Boggs-RR (mean reaction intensity of 1.33) than to CX1834-1-2 (mean reaction intensity of 3.83). The low number of individuals in the population with P_i levels either similar to CX1834-1-2 or intermediate between the parents therefore suggested that inheritance of the low phytate trait involved more than a single locus, and that the wild-type alleles were at least partially dominant over the low-phytate alleles.

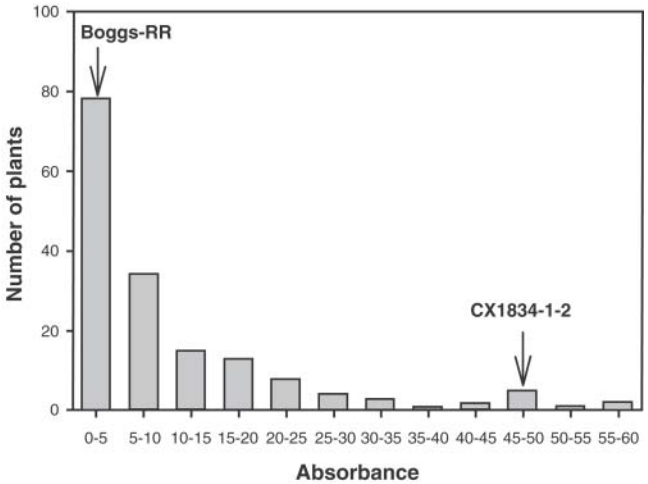

Fig. 1. Distribution of 166 F_2 plants from the Boggs-RR × CX1834-1-2 population on the basis of color intensity in P_i assays. Numbers on the X-axis represent relative P_i concentration based on absorbance measurements.

Table 1. Markers on soybean linkage groups (LGs) N and L associated with variation in P_i levels in seed from Boggs-RR × CX1834-1-2 F_2 plants.

LG	Marker	Position on LG†	Single-factor analysis of variance (P; R^2)‡	Multiple regression analysis (P; R^2)‡
N	Satt237	75.0	<0.001; 0.40	<0.001; 0.41
L	Satt561	71.4	<0.001; 0.11	<0.001; 0.11
N × L	Satt237 × Satt561	–	<0.001; 0.08	<0.001; 0.12
Total R^2:			0.59	0.64

† Positions are current estimates from the consensus linkage map of the soybean genome (Song et al., 2004).

‡ Values shown for analyses are probabilities of Type 1 errors and R^2 estimates of the portion of the phenotypic variation explained by the marker genotype or interaction between the markers.

Analysis of the SSR marker and P_i data from the Boggs-RR × CX1834-1-2 population indicated that a locus near Satt237 on LG N was associated with seed P_i levels ($P < 0.001$; $R^2 = 0.40$). Multiple regression analysis also indicated the presence of a single phytate locus on LG N (Table 1). IM and CIM analyses confirmed that this locus is close to Satt237 and most likely in the interval between Satt339 and Satt237 (Fig. 2). The importance of this locus was also supported by results from the two confirmation populations. Most of the individuals in the Benning-RR × CX1834-1-2 population subset, which was fixed for the CX1834-1-2 alleles at Satt339 and Satt237 on LG N, had intermediate to high levels of P_i compared with the two parents. In the 5601T × CX1834-1-2 population, the average P_i levels in 5601T and CX1834-1-2 seeds were 0.33 and 2.20 mg g^{-1} dry wt, respectively. Forty-three out of 44 individuals that had P_i levels ≥ 0.70 mg g^{-1} dry wt (i.e., at least double the average P_i content for 5601T) were homozygous for the CX1834-1-2 allele at Satt237.

A second locus associated with phytate levels was later identified near Satt527 and Satt561 on LG L ($P < 0.001$; $R^2 = 0.11$) by a genome-scan approach after the modified BSA method proved unsuccessful (Table 1). Single factor ANOVA, multiple regression analysis, and IM all indicated that the locus is closer to Satt561

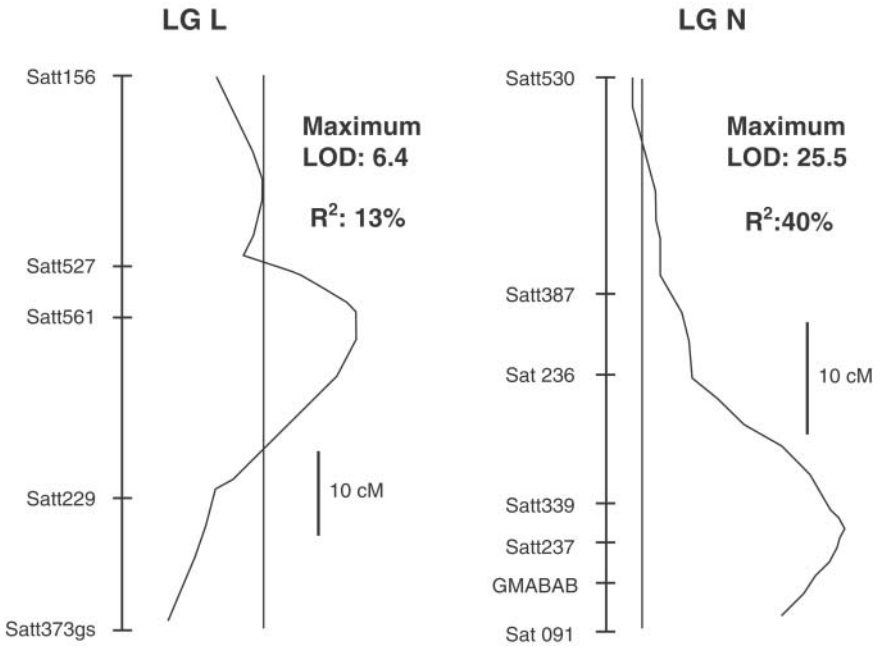

Fig. 2. LOD score plots for interval mapping on linkage groups (LGs) L and N in the Boggs-RR × CX1834-1-2 population. Lines parallel to the linkage groups indicate the genome-wide α = 0.001 significance threshold (LOD = 3.8).

(Fig. 2). Although Satt527 and Satt561 mapped 6 cM apart in the map produced from the Boggs-RR × CX1834-1-2 population, they are only 1 cM apart in the current version of the consensus map (Song et al., 2004). Although the genotype at the LG L locus had a smaller effect on the phenotype than the one on LG N, all of the progeny in the Boggs-RR × CX1834-1-2 population that had P_i levels similar to CX1834-1-2 were homozygous for the CX1834-1-2 allele at Satt561 and/or Satt527 on LG L (Fig. 3). Furthermore, the 20 RILs with the highest P_i concentrations (all >1.70 mg g^{-1}) in the 5601T × CX1834-1-2 population were all homozygous for the CX1834-1-2 allele at both Satt237 and Satt561 (Fig. 4). In the Benning-RR × CX1834-1-2 $F_{2:3}$ population, which was fixed for the CX1834-1-2 allele at Satt237 and other nearby markers on LG N, the genotype at Satt527 explained 81% of the variation in P_i content. In the 5601T × CX1834-1-2 population, 30 out of 44 individuals with P_i levels ≥ 0.70 mg g^{-1} dry wt were homozygous for the CX1834-1-2 allele at Satt561. Among individuals in this population that were homozygous for the CX1834-1-2 allele at Satt237 on LG N, the mean P_i level for those homozygous for the 5601T allele at Satt561 was 0.57 mg g^{-1} dry wt, whereas individuals

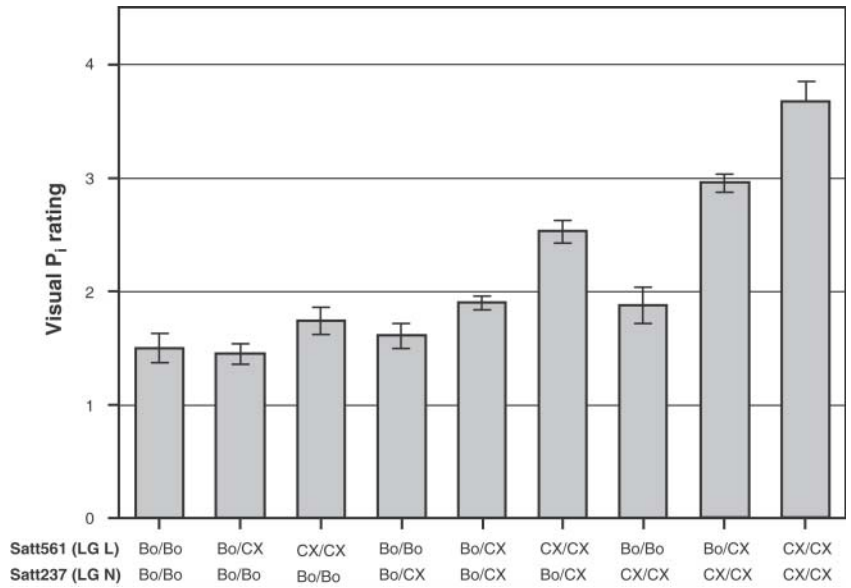

Fig. 3. P_i levels for various Satt561 (LG L) and Satt237 (LG N) marker classes in seed from a Boggs-RR × CX1834-1-2 F_2 population, based on visual ratings from colorimetric assays. (Bo = allele from Boggs-RR; CX = allele from CX1834-1-2. Visual ratings: 0 = no reaction to 5.0 = dark blue. Bars represent standard errors.)

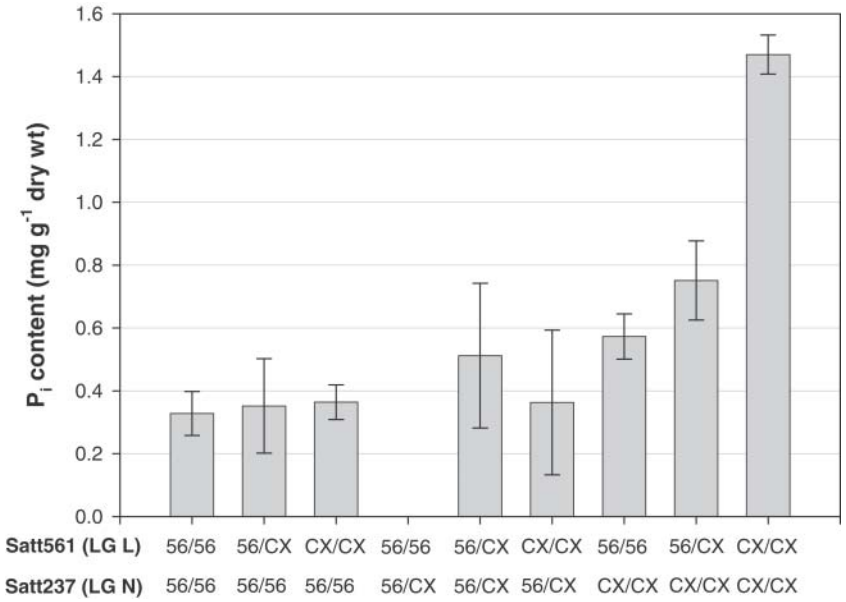

Fig. 4. P_i levels for various Satt561 (LG L) and Satt237 (LG N) marker classes in seed from a 5601T × CX1934-1-2 $F_{5:7}$ population, based on absorbance. (56 = allele from 5601T; CX = allele from CX1834-1-2. Bars represent standard errors. Note that means of classes that were heterozygous at one or both loci were calculated from only 0 to 10 individuals, whereas means of classes homozygous at both loci were calculated from 30 to 51 individuals.)

homozygous for the CX1834-1-2 allele averaged 1.47 mg g^{-1} dry wt (Fig. 4).

During the course of our investigations, reports on studies of segregation for seed phytate levels in wheat (Guttieri et al., 2004) and in independent soybean populations with the same M153 ancestor as our populations (Oltmans et al., 2004) were published. Authors of both papers also concluded that inheritance of the low phytate phenotype in their populations involved two independently segregating loci. Oltmans et al. (2004) based their conclusion on phenotypic segregation ratios in progeny tests and did not attempt to map the two loci. The phytic acid loci we detected on LGs L and N appear to be located in duplicated regions of the soybean genome, since the RFLP probes B162 and A535 anneal to sequences that flank the estimated locations of the loci on both linkage groups (Shoemaker, 2004). The two loci may therefore share a common origin, and it is possible that genes at the two loci encode related proteins. CIM analysis of our populations did not indicate the presence of additional phytic acid loci in other regions of either linkage group.

Epistasis involving the loci on LGs L and N also contributed to variance for P_i content in the Boggs-RR × CX1834-1-2 population ($P < 0.001$; $R^2 = 0.08$), as shown in Table 1. The effect of allele substitution at each locus on P_i levels depended on the genotype at the other locus, as can be seen in Fig. 3. If Satt237 on LG N was homozygous for the Boggs-RR allele, then the genotype at Satt561 on LG L had little or no effect on seed P_i levels. When Satt237 was heterozygous or homozygous for the CX1834-1-2 allele, however, substitution with a CX1834-1-2 allele at the LG L locus caused an additive increase in P_i levels. Progeny with P_i levels similar to CX1834-1-2 were homozygous for the CX1834-1-2 alleles at both of these loci, and heterozygosity at either locus resulted in intermediate P_i levels (Fig. 3). This epistatic interaction was also evident in the 5601T × CX1834-1-2 population (Fig. 4).

Oltmans et al. (2004) described the type of epistasis that they observed in populations derived from CX1834-1-6, a sister line of CX1834-1-2, as "duplicate dominant epistasis." F_1 seeds from reciprocal crosses between CX1834-1-6 and A00-711013 (normal phytate) were reported to have normal phytate levels, indicating complete dominance of the wild-type alleles. Although this appears to differ from the incomplete dominance which we observed in the Boggs-RR × CX1834-1-2 population, differences in assay methodology, scoring, and interpretation may have contributed more to the apparent discrepancy than differences in genetic backgrounds and environments. For example, Oltmans et al. (2004) classified their phenotypic reactions as either dark blue or light blue, whereas we used comparisons with a series of P_i standards in replicated assays to obtain semi-quantitative data. Furthermore, they scored their assay reactions after only 15 to 20 min, whereas we waited at least 1 h, as did Wilcox et al. (2000). This delay allowed us to observe reaction intensities that were intermediate between those of the parents. In addition, we ran our analyses on means of replicated assays from independent extractions to improve the accuracy of our P_i estimates. Lastly, the use of molecular markers allowed us to analyze our populations with a greater level of precision than would have been possible for Oltmans et al. (2004) using a classical quantitative genetics approach based on the assumption that the two loci have an equal effect on phenotype. Mean P_i levels in the double heterozygote class of our Boggs-RR × CX1834-1-2 population (equivalent to the F_1s in the population of Oltmans et al., 2004) were only marginally greater than those of the individuals homozygous for Boggs-RR alleles at the LG L and N marker loci. In any case, the

differences in our results and those of Oltmans et al. (2004) are minor compared with the similarities. We both concluded that inheritance of the low phytate trait from CX1834-1-derived lines involves two loci with an epistatic interaction, and that the low phytate phenotype equivalent to that of CX1834-1-2 is only observed in progenies that are homozygous for the CX1834-1-2 allele at both loci. We therefore propose that the names *pha1* and *pha2* designated by Oltmans et al. (2004) be henceforth applied to the CX1834-1-2 alleles at the phytate loci on LG N and LG L, respectively.

Wilcox et al. (2000) soaked about 2500 CX1515-4 seeds in an 18 m*M* EMS solution for 24 h and obtained two M2 plants (M153, the ancestor of CX1834-1-2, and M766) which produced progenies segregating for high and low P_i. The probability that EMS would have simultaneously induced two nonlethal mutations in independent phytate genes in a single seed is low, though genes from certain families appear to be more prone to acquiring mutations than others (Koornneef et al., 1982). A mutant allele conditioning low phytate may have already been present at either the LG L locus or the LG N locus in the CX1515-4 breeding line from which CX1834-1-2 is derived. Phytic acid levels among soybean lines can vary, suggesting that natural genetic variation for this trait exits (Raboy et al., 1984). If the EMS treatment had induced a mutation in only one of the two loci that we detected, the LG N locus seems the more likely candidate because of the greater effect that it has on seed P_i content. Nevertheless, mean P_i levels of the two genotype classes in the Boggs-RR × CX1834-1-2 population that were homozygous for the Boggs-RR allele at one locus and for the CX1834-1-2 allele at the other were similar (Fig. 3). It is also possible that additional undetected minor genes may influence P_i levels, since we were unable to find polymorphic markers in some regions of several linkage groups in the Boggs-RR × CX1834-1-2 population. Although the likelihood that mutations at both loci were induced by EMS is small, it cannot be dismissed without further investigation.

Hitz et al. (2002) attributed the low phytic acid phenotype in their soybean mutant to a single, recessive mutation. The approximately 50% reduction in phytic acid in the seeds of the mutants was accompanied by an increase in the level of P_i and by a substantial decrease in both raffinose and stachyose in the most highly characterized lines. In contrast, the mutation in the M153-1-4 ancestor of CX1834-1-2 did not alter the concentrations of these polysaccharides. Relative sucrose, raffinose, and stachyose content in low and high P_i lines derived from M153-1-4 were similar and were unrelated to P_i levels in those lines (data not shown). While these results show that the mutations are not identical, they do not indicate whether the mutations involve different genes, or different regions of the same gene. Hitz et al. (2002) used *N*-nitroso-*N*-methylurea rather than EMS as a mutagen. The multiple phenotypic changes observed by those authors resulted from a single base change in a *myo*-inositol 1-phosphate synthase gene which causes the substitution of a lysine residue with asparagine. This substitution results in a 90% decrease in the specific activity of a *myo*-inositol 1-phosphate that is involved in phytic acid synthesis. Although there is no reported evidence for raffinose and stachyose synthase genes on LGs L and N, a gene determining fucose levels in the cell walls of both whole seed and soybean embryos has been mapped near the phytate locus on LG L (Stombaugh et al., 2004).

The phytate locus on LG L resides in a region to which QTLs associated with a number of seed traits have been mapped, including seed weight and seed yield (Orf et al., 1999a, 1999b; Stombaugh et al., 2004), fatty acid composition (Hyten et al., 2004a; Maria Monteros, personal communication), and protein (Orf et al., 1999a). Satt561 is also linked to QTLs affecting plant height, flowering date (R1), and maturity date (R8) (Orf et al., 1999b). Fewer seed trait QTLs have been mapped to the Satt237/Satt339 region of LG N, but Kabelka et al. (2004) reported QTLs for seed protein and seed yield in that region. QTLs associated with iron deficiency chlorosis (Lin et al., 1997) and salt tolerance (Lee et al., 2004) have been mapped to both this region of LG N and to the region of the phytate locus on LG L. This may simply reflect the duplicated nature of the regions on these two linkage groups, but the fact that phytic acid chelates iron makes the coincidence intriguing.

Digenic inheritance of low seed phytate levels in populations derived from CX1834-1-2 will make selection for this trait less efficient than it would be if only one locus were involved. Only about 6% of the individuals in a segregating F_2 population can be expected to have the low phytate phenotype. Breeders attempting to select low phytate individuals that also carry favorable alleles for other traits segregating in the population will need larger population sizes to maintain a high probability of finding plants with favorable allele combinations for all or most of the traits. The cluster of seed trait QTLs around Satt527 and Satt561 on LG L could cause a problem if the low phytate allele there is linked in repulsion with desirable alleles at nearby loci. This potential problem will be reduced, however, after the low phytate alleles have been introgressed into a wider variety of elite donor parents carrying favorable recombinations of alleles on LG L. The option of using MAS instead of phenotypic selection should facilitate transfer of the low phytate trait into elite lines, since the assay reaction of double heterozygotes (i.e., individuals heterozygous at both of the phytate loci) is only slightly more intense than that of the single heterozygote classes or the homozygous wild type (Fig. 3). As a result, the assay reactions of double heterozygotes (which would be obtained through backcrossing, for example) may not be distinct from those of individuals homozygous for a non-CX1834-1-2 allele at one locus, especially if assays are run on P_i extracts from small tissue samples (such as quarter-seed chips).

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